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Abstract: The directional growth of the pollen tube from the stigma to the embryo sac in the ovules is regulated by pollen-pistil interactions based on intercellular communication. Although pollen tube growth is regulated by the cytoplasmic Ca(2+) concentration ([Ca(2+)](cyt)), it is not known whether [Ca(2+)](cyt) is involved in pollen tube guidance and reception. Using Arabidopsis expressing the GFP-based Ca(2+)-sensor yellow cameleon 3.60 (YC3.60) in pollen tubes and synergid cells, we monitored Ca(2+) dynamics in these cells during pollen tube guidance and reception under semi-in vivo fertilization conditions. In the pollen tube growing towards the micropyle, pollen tubes initiated turning within 150 m of the micropylar opening; the [Ca(2+)](cyt) in these pollen tube tips was higher than in those not growing towards an ovule in assays with myb98 mutant ovules, in which pollen tube guidance is disrupted. These results suggest that attractants secreted from the ovules affect Ca(2+) dynamics in the pollen tube. [Ca(2+)](cyt) in synergid cells did not change when the pollen tube grew towards the micropyle or entered the ovule. Upon pollen tube arrival at the synergid cell, however, [Ca(2+)](cyt) oscillation began at the micropylar pole of the synergid, spreading towards the chalazal pole. Finally, [Ca(2+)](cyt) in the synergid cell reached a maximum at pollen tube rupture. These results suggest that signals from the pollen tube induce Ca(2+) oscillations in synergid cells, and that this Ca(2+) oscillation is involved in the interaction between the pollen tube and synergid cell.

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Cytoplasmic Ca^{2+} changes dynamically during the interaction of the pollen tube with synergid cells

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SUMMARY

The directional growth of the pollen tube from the stigma to the embryo sac in the ovules is regulated by pollen-pistil interactions based on intercellular communication. Although pollen tube growth is regulated by the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$), it is not known whether $[Ca^{2+}]_{cyt}$ is involved in pollen tube guidance and reception. Using *Arabidopsis* expressing the GFP-based Ca^{2+}-sensor yellow cameleon 3.60 (YC3.60) in pollen tubes and synergid cells, we monitored Ca^{2+} dynamics in these cells during pollen tube guidance and reception under semi-in vivo fertilization conditions. In the pollen tube growing towards the micropyle, pollen tubes initiated turning within 150 μm of the micropylar opening; the $[Ca^{2+}]_{cyt}$ in these pollen tube tips was higher than in those not growing towards an ovule in assays with *myb98* mutant ovules, in which pollen tube guidance is disrupted. These results suggest that attractants secreted from the ovules affect Ca^{2+} dynamics in the pollen tube. $[Ca^{2+}]_{cyt}$ in synergid cells did not change when the pollen tube grew towards the micropyle or entered the ovule. Upon pollen tube arrival at the synergid cell, however, $[Ca^{2+}]_{cyt}$ oscillation began at the micropylar pole of the synergid, spreading towards the chalazal pole. Finally, $[Ca^{2+}]_{cyt}$ in the synergid cell reached a maximum at pollen tube rupture. These results suggest that signals from the pollen tube induce Ca^{2+} oscillations in synergid cells, and that this Ca^{2+} oscillation is involved in the interaction between the pollen tube and synergid cell.

KEY WORDS: Ca^{2+} dynamics, Pollen tube guidance, Pollen-synergid interaction, *Arabidopsis*

INTRODUCTION

Sexual reproduction in flowering plants starts when a pollen grain lands on the surface of the stigma, hydrates and germinates a pollen tube. The pollen tube enters the style and grows through the transmitting tract tissue to the ovary, where it is guided along the funiculus and enters the micropyle of the ovule to deliver two sperm cells to the embryo sac to effect double fertilization. This directional growth of the pollen tube from the stigma to the embryo sac is controlled by cellular interactions, and Ca^{2+} is thought to be a key player in regulating these processes (Dumas and Gaude, 2006; Chae and Lord, 2011).

Pollen tube guidance by the embryo sac involves at least two steps (Shimizu and Okada, 2000). First, funicular guidance directs the pollen tube from the placenta along the funiculus to the ovule. This is followed by micropylar guidance, which guides the pollen tube into the micropyle to reach the embryo sac (reviewed by Márton and Dresselhaus, 2010; Takeuchi and Higashiyama, 2011). Electron microscopic studies showed that the synergid cells and the filiform apparatus, a structure at their micropylar pole, contain high Ca^{2+} concentrations ($[Ca^{2+}]$) (Chaubal and Reger, 1990; Chaubal and Reger, 1992a; Chaubal and Reger, 1992b; Chaubal and Reger, 1993). In *Arabidopsis*, the transcription factor MYB98 is expressed in synergid cells and required for development of the filiform apparatus and micropylar guidance, suggesting a role of the filiform apparatus in this process (Kasahara et al., 2005; Punwani et al., 2007). In addition, in vitro assays of pollen tube chemotropism towards excised pistil tissue and various compounds suggested that Ca^{2+} might be a potential attractant (Mascarenhas and Machlis, 1962; Reger et al., 1992). In *Torenia fournieri*, however, elevation of $[Ca^{2+}]$ did not affect pollen tube attraction in in vitro guidance assays (Higashiyama et al., 2006). In this system, it was unambiguously shown that the synergids produce pollen tube attractants (Higashiyama et al., 2001). Recently, the *Torenia* LURE proteins, secreted cysteine-rich peptides that accumulate in the filiform apparatus of the synergid cells, have been identified as pollen tube attractants (Okuda et al., 2009; Okuda and Higashiyama, 2010). In *Arabidopsis*, synergids and other embryo sac cells produce a diverse set of cysteine-rich proteins (Jones-Rhoades et al., 2007; Dresselhaus and Márton, 2009; Wuest et al., 2010). This indicates that similar attractants produced by the synergid cells also exist in *Arabidopsis*, although their molecular identity is not yet known.

However, it is not clear whether Ca^{2+} in the synergid cells is relevant to pollen tube guidance and reception in *Arabidopsis*. On the other hand, the auto-inhibited Ca^{2+} ATPase (ACA) ACA9, which is predicted to be activated by Ca^{2+}/calmodulin, is expressed primarily in the plasma membrane of the *Arabidopsis* pollen tube, and disruption of *ACA9* results in partial male sterility. In an *aca9* mutant, pollen tubes normally reach the embryo sac and cease growth, but they fail to rupture and release the sperm cells (Schiøtt et al., 2004). This finding suggests the involvement of Ca^{2+} signaling in sperm discharge. The relationship between $[Ca^{2+}]$ and pollen tube growth has previously been examined in vitro, where pollen tubes germinate and grow in culture medium. Ratiometric ion imaging using fluorescent dyes has revealed that the apical domain of a pollen tube grown in vitro contains a tip-focused $[Ca^{2+}]$ gradient (Pierson et al., 1994; Pierson et al., 1996; Cheung and Wu, 2008). Genetically encoded Ca^{2+} indicators, yellow cameleons (YCs), have been

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developed to monitor [Ca^{2+}] in living cells (Miyawaki et al., 1997; Nagai et al., 2004). Using plants expressing YC3.1 or YC3.60, Ca^{2+} dynamics in the pollen grain, pollen tub, and stigmatic papillar cell were monitored during pollination, as well as on pollen germination medium (Iwano et al., 2004; Watahiki et al., 2004; Iwano et al., 2009). These studies revealed that cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$) in the tip region of the growing pollen tube are maintained at submicromolar levels under all conditions.

Imaging of micropylar guidance has been performed under a semi-in vivo fertilization system in *Torenia* and *Arabidopsis* (Higashiyama et al., 1998; Palanivelu and Preuss, 2006; Higashiyama and Hamamura, 2008; Hamamura et al., 2011). In *Arabidopsis*, labeling the cytoplasm of the synergid cells and pollen tubes with GFP and/or RFP revealed that a pollen tube emerging from a cut style grows towards excised ovules, enters the ovule through the micropyle, ceases growth at the synergid cells and ruptures to release the sperm cells (Sandaklie-Nikolova et al., 2007). Furthermore, labeling of sperm cell nuclei with mRFP and labeling of the synergid, egg and central cells with GFP have enabled the successful study of double fertilization (Ingouff et al., 2007; Hamamura et al., 2011).

Here, to examine Ca^{2+} dynamics during micropylar guidance and pollen tube reception, we employed *Arabidopsis* plants expressing the Ca^{2+} sensor YC3.60 in pollen tubes and synergid cells and monitored Ca^{2+} dynamics in these cells during semi-in vivo fertilization. We set up a monitoring system using an EM-CCD camera attached to a wide-field microscope to follow Ca^{2+} dynamics and found remarkable changes in $[Ca^{2+}]_{cyt}$ both in the pollen tube and the synergid cells, suggesting an involvement of Ca^{2+} in the communication between these cells. We discuss our results in relation to the physiological relevance of $[Ca^{2+}]_{cyt}$ for the interactions between the pollen tube and synergid cell during micropylar pollen tube guidance and pollen tube reception.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana accession Columbia was used for generation of transgenic plants. Plants were grown in pots containing soil/perlite (3:1, by volume) in a growth chamber with a light intensity of 120-150 mmol m^{-2} s^{-1} during the daily 12-hour light period. The temperature was maintained at 22±2°C.

Vector construction

A DNA fragment encoding DsRed was amplified from pDsRed2 vector (Clontech) by PCR using the following primers: 5′-GCTCTAGATGGCCTCCTCCGAGAACGT-3′ and 5′-GTGCGAGCTCTACAGGAACAGGTGGTGGC-3′; the incorporated *Xba*I and *Sac*I sites are underlined. The obtained DNA fragment was digested with *Xba*I and *Sac*I, and then ligated to the *Xba*I and *Sac*I sites of the pBI121-pAct1::YC4.60(ER) vector used previously (Iwano et al., 2009), yielding the pBI121-pAct1::RFP vector.

For the constructs expressing YC3.60 in the synergid cells, promoter regions of *DD2* (*At5g43510*) and *DD2-like* (*At5g43513*) genes were amplified by PCR with primers (5′-CTTAAGCTTTCTCTGTTTCTTATCAG-3′ and 5′-CCATGGTTCTTGGTAGAAATTAACCAATATCAC-3′ for *DD2* promoter; 5′-AAGCTTGAGTTTGGATTGATTCGAGGC-3′ and 5′-CCATGGTTCTTTGTAGAAATTAACCAATATCAC-3′ for *DD2-like* promoter; the incorporated *Hind*III and *Nco*I sites are underlined). Obtained fragments were digested with *Hind*III and *Nco*I and ligated to *Hind*III and *Nco*I sites of pLat52::YC3.60 previously constructed (Iwano et al., 2009) for yielding pDD2::YC3.60 and pDD2-like::YC3.60.

Transformation

The pBI121-Act1::RFP plasmid, pDD2::YC3.60 plasmid and pDD2-like::YC3.60 plasmid were electroporated into *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993). The *Agrobacterium* infiltration procedure was performed with unopened flower buds of *Arabidopsis* accession Columbia as previously described (Iwano et al., 2004). Transformed seeds were selected on 1/2 MS plates containing kanamycin (50 μg/ml) and were analyzed using PCR to test for the presence of the *RFP* (*DsRed*) gene.

Pollen tube growth, ratiometric imaging and image analysis

For imaging under the semi-in vivo condition, wild-type *Arabidopsis* flowers excised before anther dehiscence were collected onto an agar plate and the anthers were removed. Pollen grains from freshly dehisced anthers of pAct1::YC3.60-expressing plants were loaded onto the wild-type stigma. Thirty minutes after manual pollination, the upper part of the pollinated pistil was excised and mounted in the germination medium (Boavida and McCormick, 2007) in a moistened glass-bottomed dish. Next, following previously reported methods (Palanivelu and Preuss, 2006), pAct1::YC3.60- or pDD2like::YC3.60-expressing ovules were excised from transgenic *Arabidopsis* plants. After 2 hours at 23°C, the $[Ca^{2+}]_{cyt}$ in pollen tubes growing through the style and towards the synergid cells during micropylar guidance was imaged with a Zeiss Axio Observer inverted microscope equipped with a Xenon lamp, an EM-CCD Evolve 512 camera (Photometrics) and a filter exchanging system (Prior). Imaging of the cameleon emission ratio was accomplished using two emission filters (480/30 for ECFP, 535/40 for Venus and 641/75 for DsRed). After background subtraction, the Venus/ECFP ratio was determined using MetaMorph software (Molecular Devices, USA). A Zeiss C Apo 40×/1.20 water immersion objective lens and a Plan-Apo 20×/0.8 objective lens were used for imaging. Exposure times were typically 20 mseconds, and images were collected every 3-20 seconds. In each experiment, the ratio in a region of interest of 6 × 6 μm^2 was measured using the MetaMorph software, and is shown as sequential line graphs. The mean and maximum values were calculated using Microsoft Excel.

To confirm that the full-length cameleon was expressed in the pollen tube, elongated pollen tubes were monitored with excitation at 442 nm using a spectral imaging fluorescence microscope system (LSM710 META, Carl Zeiss, Jena, Germany). This system is capable of resolving the spectra of various fluorescence images; therefore, we were able to obtain images with no interference from the overlapping fluorescence emissions (Iwano et al., 2009). These experiments were carried out more than 30 times.

Calibration of YC3.60 ratiometric changes

Calibration of the $[Ca^{2+}]_{cyt}$ was carried out as described previously (Allen et al., 1999). Serial dilutions of purified YC3.60 were made in Ca^{2+} calibration buffer (Molecular Probes), in which the free [Ca^{2+}] ranged from 0 mM to 1 mM. Dilutions of YC3.60 that resulted in similar signal intensities to those seen in YC3.60-expressing pollen tubes were used to determine the minimum (R_{min}) and maximum (R_{max}) ratio values. For YC3.60, R_{min} and R_{max} values were 1.75 and 5.35 (supplementary material Fig. S1). These values were used to convert the YC3.60 fluorescence ratios into a $[Ca^{2+}]_{cyt}$ by fitting them to YC3.60 calibration curves obtained in vitro.

Statistical analysis

Statistical analyses were performed using Student's *t*-test where necessary.

RESULTS

Ca^{2+} imaging in pollen tube tips growing in the presence and absence of functional ovules

$[Ca^{2+}]_{cyt}$ in pollen tubes growing under in vitro and semi-in vivo conditions has been examined in previous studies (Iwano et al., 2004; Iwano et al., 2009). However, it remained unclear whether ovules affect $[Ca^{2+}]_{cyt}$ dynamics in the pollen tube. First, we examined whether growth and $[Ca^{2+}]_{cyt}$ in pollen tubes changes in the presence of ovules. In these experiments, wild-type ovules were placed about 450 μm away from the pistil excision site. After incubation of the pollinated pistil for ~2 hours at 23°C, pollen tubes emerged from the pistil excision site. The $[Ca^{2+}]_{cyt}$ in pollen tubes

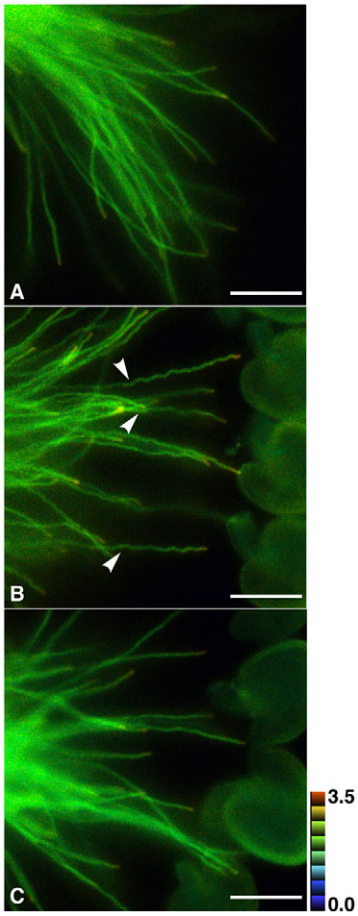

Fig. 1. Ratio images of pAct1::YC3.60-expressing pollen tubes growing out of excised pistils. (**A**) Pollen tubes growing in the absence of ovules. (**B**) Pollen tubes growing toward wild-type ovules. Arrowheads indicate where the turning of the pollen tube started. (**C**) Pollen tubes growing in the presence of *myb98* ovules. Scale bars: 100 μm.

were monitored at intervals of 10 seconds using a 20× objective lens. As a result, 75% of ovules successfully attracted pollen tubes, which entered the micropyle and stopped growth at the synergid cells (18/24 samples).

For comparison, excised pollinated pistils were placed on the pollen growth medium without ovules and monitored. In this case, pollen tubes grew straight, even when their lengths were over 400 μm out of excised pistils (Fig. 1A). However, in the presence of ovules, pollen tubes grew straight until they were ~150 μm from a micropyle, then grew in a wave-like pattern towards an ovule (Fig. 1B; supplementary material Movie 1). To elucidate whether the wave-like turning of the pollen tub was related to synergid function, ovules from the *myb98* mutant, in which the formation of the filiform apparatus and pollen tube guidance are defective, were placed on the growth medium. In this case, pollen tubes grew straight and only rarely grew into a micropyle (1/29) (Fig. 1C; supplementary material Movie 2).

Next, we monitored the $[Ca^{2+}]_{cyt}$ in pollen tubes growing towards the micropyles of wild-type and *myb98* ovules, or without ovules. When the distance from the micropyles of wild-type ovules was about 30-70 μm, the average Venus/ECFP ratio in the tip was 3.18±0.48 (n=18) (Fig. 2A1-A3). Especially, when the pollen tube was turning towards a micropyle, a broader and stronger $[Ca^{2+}]_{cyt}$ signal at the pollen tip was observed (Fig. 2B). By contrast, in pollen tubes growing within 70 μm of the micropyles of *myb98* ovules, the $[Ca^{2+}]_{cyt}$ did not change when the pollen tubes approached the micropyles more closely by chance and the average ratio was 2.10±0.16 (n=10), which was significantly different from that in the presence of wild-type ovules (P<0.001) (Fig. 2C,D). Furthermore, in the pollen tubes growing out of a cut style without wild-type ovules, the average ratio was 1.98±0.15 (n=10), which was significantly different from that in the presence of wild-type ovules (P<0.001), but not in the presence of *myb98* mutant ovules (Fig. 2C-E). These results were reproducibly obtained in the experiments using another pAct1::YC3.60-expressing line (wild-type ovule, 3.30±0.32; *myb98* ovule, 2.21±0.31; without ovule, 2.07±0.21).

To convert the YC3.60 ratios into approximate $[Ca^{2+}]_{cyt}$ values, calibration of $[Ca^{2+}]_{cyt}$ was carried out as described previously (Allen et al., 1999; Iwano et al., 2009). We derived a titration curve (supplementary material Fig. S1, see Materials and methods), from which we estimated that the $[Ca^{2+}]_{cyt}$ in the tip region shown in Fig. 2 ranged from 0.2 to 0.7 μM in the presence of wild-type ovules and from 0.2 to 0.3 μM in the presence of *myb98* ovules or in the absence of ovules.

Furthermore, in order to examine whether $[Ca^{2+}]_{cyt}$ in the pollen tip increases just after emergence from the pistil or gradually during its approach to the ovules, the ratio in the pollen tubes emerging from the pistils was monitored every 3-5 minutes over a range of 150 to 400 μm from the pistil excision site, the ovules being 450 to 500 μm away. Without ovules and in the presence of *myb98* mutant ovules, there was no obvious change in the ratio (Fig. 2F). In the presence of wild-type ovules, the ratio was no different from that without ovules or with the *myb98* ovules in the range of 150-200 μm, but increased between ~250-300 μm, where the pollen tubes started to turn; the increase continued at ~350-400 μm (Fig. 2F). These observations indicate that $[Ca^{2+}]_{cyt}$ in the pollen tip increases as the pollen tubes approach the ovules, before it decreases as the tubes grow closer to the micropyle. Collectively, these results suggest that $[Ca^{2+}]_{cyt}$ dynamics in the pollen tube changes in response to ovules, especially synergid cells, supporting our hypothesis that Ca^{2+} signaling in pollen tubes is involved in micropylar guidance. Moreover, as $[Ca^{2+}]_{cyt}$ in pollen tubes increases at some distance in the presence of wild-type but not *myb98* ovules, functional synergids are also involved in producing long-range effects on $[Ca^{2+}]_{cyt}$ in the pollen tube.

Expression of YC3.60 in synergid cells

Previously, eight independent YC3.60-expressing plants were obtained by transformation with the pAct1::YC3.60 plasmid (Iwano et al., 2009). Surprisingly, one of these transgenic lines expressed YC3.60 not only in the pollen grain and pollen tube, but also in the synergid cells, and to a lesser degree the central cell of the embryo sac and the sporophytic tissues of the ovule (supplementary material Fig. S2A,B). Seed set in this transgenic line (51.8±5.2 seeds, n=30) was no different from that of wild-type plants (50.9±6.7 seeds, n=30). In particular, the intensity of YC3.60 in the synergid cells was the same as that in the pollen tube. To confirm that the synergid cells expressed the ECFP and Venus components of YC3.60, we obtained fluorescence spectra from these cells using a spectral-imaging microscope system with excitation at 442 nm. The YC3.60 spectrum was a combination of the spectra typically observed for recombinant ECFP and Venus proteins. Photobleaching the synergid cells with a 514 nm light induced ~90% increase in ECFP fluorescence (supplementary material Fig. S2C). However, as the expression of the *ACTIN1*

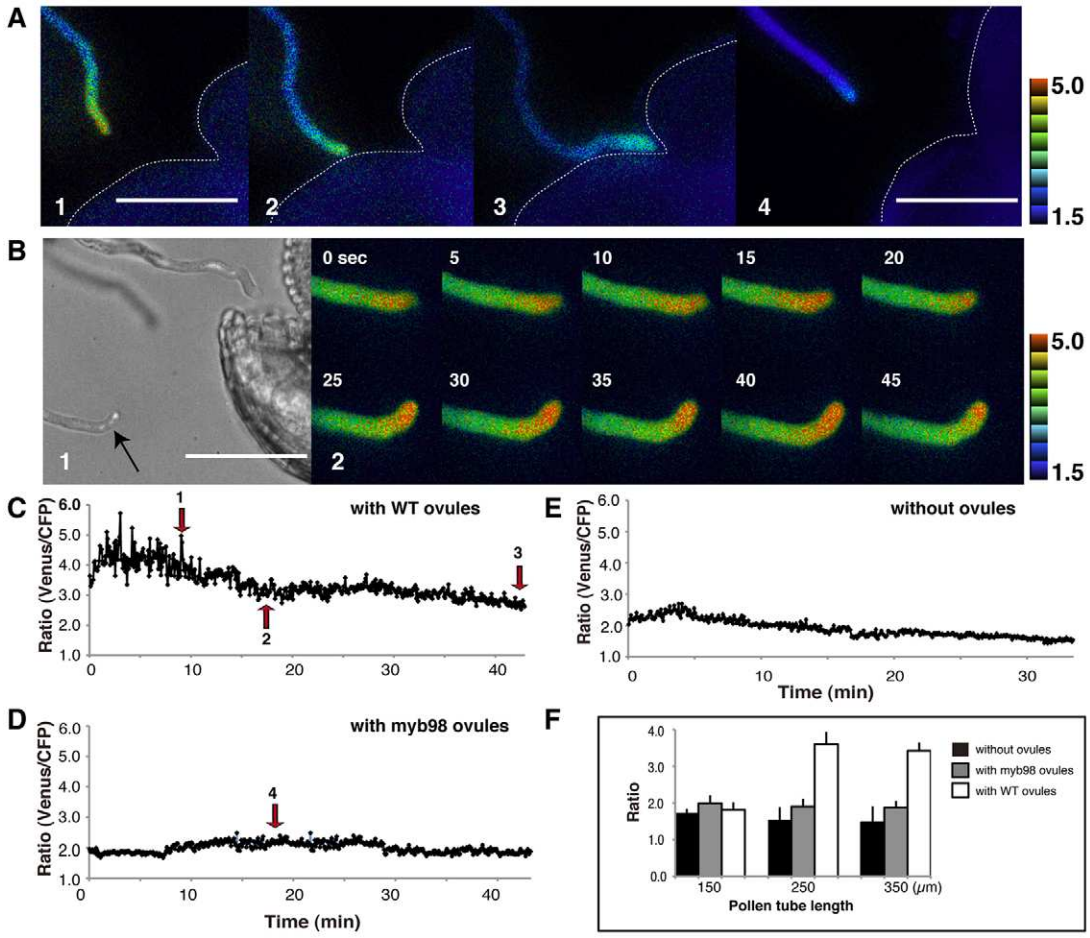

Fig. 2. Ratio images of pAct1::YC3.60-expressing pollen tubes growing in the presence and absence of functional ovules. (**A**) Ratio images of the pollen tubes growing towards a wild-type ovule (1-3) and in the presence of a *myb98* ovule (4). Numbers in the images correspond to the numbers in the graphs in C and D. (**B**) Ratio changes (2) of the pollen tube tip (1, arrow) turning towards a wild-type ovule. Images were captured at 5-second intervals. Scale bars: 50 μm. (**C**) Ratio changes of a pollen tube tip growing in the presence of a wild-type ovule. At time 0, the pollen tube was 65 μm from the micropyle. (**D**) Ratio changes of a pollen tube tip growing in the presence of a *myb98* ovule. At time 0, the pollen tube was 65 μm from the micropyle. (**E**) Ratio changes of a pollen tube tip growing without ovules. The pollen tube length is about 350 μm from the pistil excision site. (**F**) Average of ratios of pollen tube tips growing without ovules and in the presence of *myb98* ovules and wild-type ovules. 'Pollen tube length' means the length of the pollen tube elongated from the pistil excision site. Data are mean±s.e.m.

gene has not previously been reported in synergid cells, several lines expressing YC3.60 in synergids were made using promoters of the *DD2* gene expressing specifically in synergid cells (Steffen et al., 2007) and of a paralogous gene (*DD2-like*). We obtained two lines for pDD2::YC3.60 and four lines for pDD2-like::YC3.60. The YC3.60 protein was specifically expressed in synergid cells in both pDD2-like::YC3.60 lines, and two synergid cells could be distinguished under confocal microscopy (supplementary material Fig. S2D-F); expression in the pDD2::YC3.60 lines was similar but low. Seed sets in these transgenic lines (48.5±6.5 seeds, 49.1±7.0 seeds, n=30) were not different from that of wild-type plants (50.9±6.7 seeds, n=30). Therefore, in this study, two distinct YC3.60 lines, pAct1::YC3.60 and pDD2-like::YC3.60, were used.

$[Ca^{2+}]_{cyt}$ changes in both the pollen tube and synergid cells during micropylar guidance and pollen tube rupture

In order to examine Ca^{2+} dynamics in the pollen tube and synergid cells during the interaction between these two gametophytic cells, we simultaneously monitored their $[Ca^{2+}]_{cyt}$ dynamics using ovules with pAct1::YC3.60-expressing synergid cells in the semi-in vivo fertilization system described above. In these experiments, ovules were placed 300 μm from the excised edge of the pistil.

First, $[Ca^{2+}]_{cyt}$ in the synergid cells was monitored under a 20× objective before and after pollen tube arrival (Fig. 3). At intervals of 3 to 10 seconds, pollen tubes terminated at 90% of the synergid cells (10/11 samples), and 60% of the pollen tubes that reached the synergid cells burst (6/10 samples).

Next, both the pollen tube and the synergid cells inside the ovule were observed under a 40× objective (data not shown). $[Ca^{2+}]_{cyt}$ dynamics were monitored in these cells as the pollen tube approached at intervals of 3 to 20 seconds. Pollen tubes terminated at about 75% of the synergid cells (9/12 samples), and 55% of the pollen tubes that reached the synergid cells burst (5/9 samples). Before the approach of the pollen tube, the Venus/ECFP ratios of synergid cells were 1.96±0.06 (n=5), and $[Ca^{2+}]_{cyt}$ was estimated at about 0.1 μM (supplementary material Fig. S3); there was no significant $[Ca^{2+}]_{cyt}$ change over time. When a pollen tube arrived at the synergid cells, however, $[Ca^{2+}]_{cyt}$ in the synergid cell increased in the area next to the point of the pollen tube contact

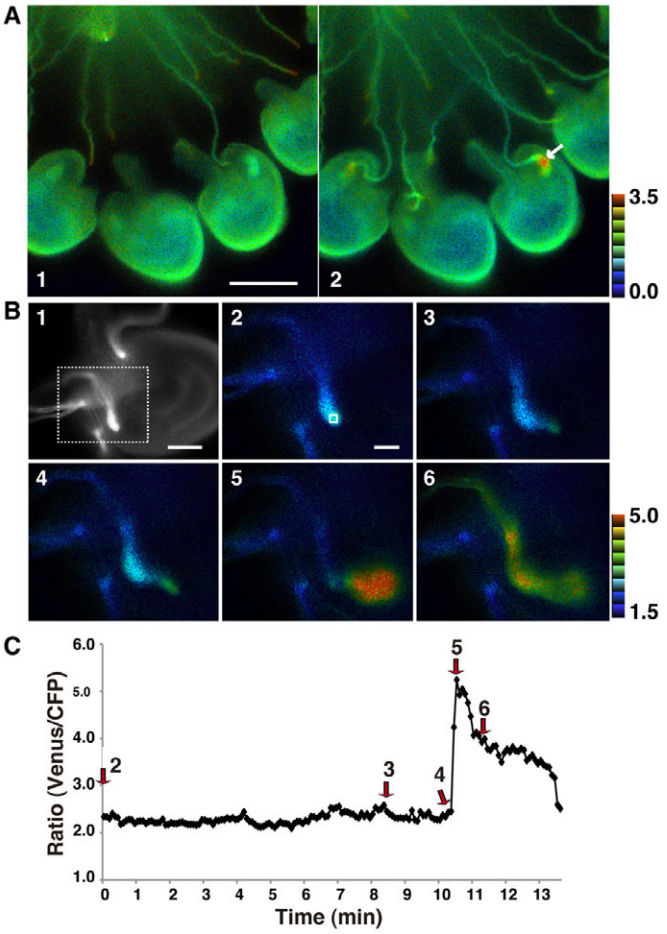

Fig. 3. Ca^{2+} imaging in the pollen tubes and synergid cells during micropylar guidance and pollen tube rupture. pAct1::YC3.60-expressing plant was used. (**A**) (1) Ratio image before pollen tubes arrived at the synergid cells. (2) Ratio image after pollen tube arrival. The $[Ca^{2+}]_{cyt}$ increased in the synergid cells (white arrow). Scale bar: 100 μm. (**B**) Ratio images in the pollen tube from cessation of growth to tube rupture. (1) a Venus image of an ovule with an arriving pollen tube. (2) Higher magnification images of the area outlined in 1. The square in 2 indicates pollen tube tip. Scale bars: 50 μm in 1; 10 μm in 2-6. (**C**) Ratio changes of the region of interest in the tip area of the pollen tube shown in B. Numbers in the graph correspond to each image number in B.

(supplementary material Fig. S3B, image number 2). After the pollen tube grew past the synergid cell, $[Ca^{2+}]_{cyt}$ in the entire synergid cell area further increased (supplementary material Fig. S3B, image number 3). Finally, $[Ca^{2+}]_{cyt}$ of the synergid cells increased strongly at the time of pollen tube rupture; however, because both the synergid cells and pollen tubes express the cameleon, it is difficult to determine the exact origin of the signal. On the pollen tube side, the Venus/ECFP ratio of the pollen tube tip growing within the ovule was 2.54±0.26 (n=5, converted to a $[Ca^{2+}]_{cyt}$ of 0.2-0.3 μM), and there was no significant change over time. However, pollen tube tip $[Ca^{2+}]_{cyt}$ increased substantially at the time of pollen tube burst (supplementary material Fig. S3B).

In all experiments under 20× and 40× objective, although we could observe the $[Ca^{2+}]_{cyt}$ dynamics of the pollen tube and the synergid cells during micropylar guidance, it was difficult to distinguish the change of $[Ca^{2+}]_{cyt}$ in the synergid cells from that of the pollen tube after the pollen tube had arrived at the synergid cells because they spatially overlapped. Therefore, to resolve the $[Ca^{2+}]_{cyt}$ dynamics in these cells, we monitored $[Ca^{2+}]_{cyt}$ separately in either only the pollen tube or only the synergid cells (see below).

Ca^{2+} dynamics in pollen tubes during micropylar guidance and pollen tube reception

We observed $[Ca^{2+}]_{cyt}$ changes in pAct1::YC3.60-expressing pollen tubes growing towards and inside wild-type ovules (Fig. 3B,C; supplementary material Movie 3). After entering the micropyle and approaching the synergid cells, pollen tube growth ceased temporarily and pollen tube tips swelled (Fig. 3B1,B2). Pollen tubes later resumed growth (Fig. 3B3,B4) and ruptured shortly afterward (Fig. 3B5,B6). Interestingly, between the time of growth cessation and rupture, $[Ca^{2+}]_{cyt}$ at the pollen tube tip did not significantly change and the tip-focused $[Ca^{2+}]_{cyt}$ gradient was prominent, except for a slight elevation of $[Ca^{2+}]_{cyt}$ in the shank region of the pollen tube (Fig. 3B3,B4). However, $[Ca^{2+}]_{cyt}$ increased from a ratio of 2.5 to 5 (0.15 μM to >1 μM) at the time of pollen tube rupture. In bursting pollen tubes, an area of high $[Ca^{2+}]_{cyt}$ rapidly spread from the tip to the shank. These $[Ca^{2+}]_{cyt}$ changes were observed in all experiments (3/3).

Ca^{2+} dynamics in the synergid cells during micropylar guidance and pollen tube reception

In order to observe the precise $[Ca^{2+}]_{cyt}$ change in the synergid cells alone, we monitored $[Ca^{2+}]_{cyt}$ dynamics in the pAct1::YC3.60-expressing synergid cells that were approached by RFP-expressing pollen tubes (Fig. 4; supplementary material Movie 4). When the pollen tubes made contact with the synergid cells, they temporarily ceased growth, and a Ca^{2+} oscillation was initiated at the contact point near the micropylar end of the synergid cells (Fig. 4A1,A2; 4B1,B2) and subsequently spread throughout the cell (Fig. 4A3; 4B3). When the pollen tubes grew further, the amplitude of the oscillation eventually decreased (Fig. 4A4,B4), but $[Ca^{2+}]_{cyt}$ increased again at about 5 minutes before pollen tube rupture (Fig. 4A5,B5). Finally, the $[Ca^{2+}]_{cyt}$ in the synergid cells reached a maximum (ratio=3.99±1.06; concentration=0.7 μM, n=4) when the pollen tube ruptured and decreased afterwards (Fig. 4B5). These $[Ca^{2+}]_{cyt}$ changes were also observed in all experiments using plants expressing pDD2-like::YC3.60 (3/3) (maximum ratio=4.08±1.05; concentration=0.75 μM, n=3) and indicate that physiological changes in the synergid cells during pollen tube guidance and reception involve $[Ca^{2+}]_{cyt}$ dynamics.

Upon closer examination, we detected some variation in the timing of the appearance of Ca^{2+} oscillation in the synergids after the pollen tubes enter the micropyle. Of the 10 pAct1::YC3.60 synergids and 30 pDD2-like::YC3.60 synergids monitored at intervals of 5 seconds, $[Ca^{2+}]_{cyt}$ in the synergid cells increased after a pollen tube contacted them (8/10, 26/30) (Fig. 5A,B1). But in some synergids (2/10, 4/30), a Ca^{2+} elevation was observed before pollen tube contact when it was still 10 μm to 30 μm away from the synergid cell (Fig. 5A,B2). These results suggest the possible existence of diffusible signals from the pollen tube. However, the level of Ca^{2+} elevation before contact was lower and the interval of oscillation was longer than that after contact. In addition, $[Ca^{2+}]_{cyt}$ increased strongly only after pollen tube contact. These results suggested that pollen tube contact with the synergid is important for Ca^{2+} oscillation.

DISCUSSION

Using recently developed imaging techniques, micropylar guidance in *Arabidopsis* has been visualized in a semi-in vivo fertilization system (Palanivelu and Preuss, 2006; Ingouff et al., 2007;

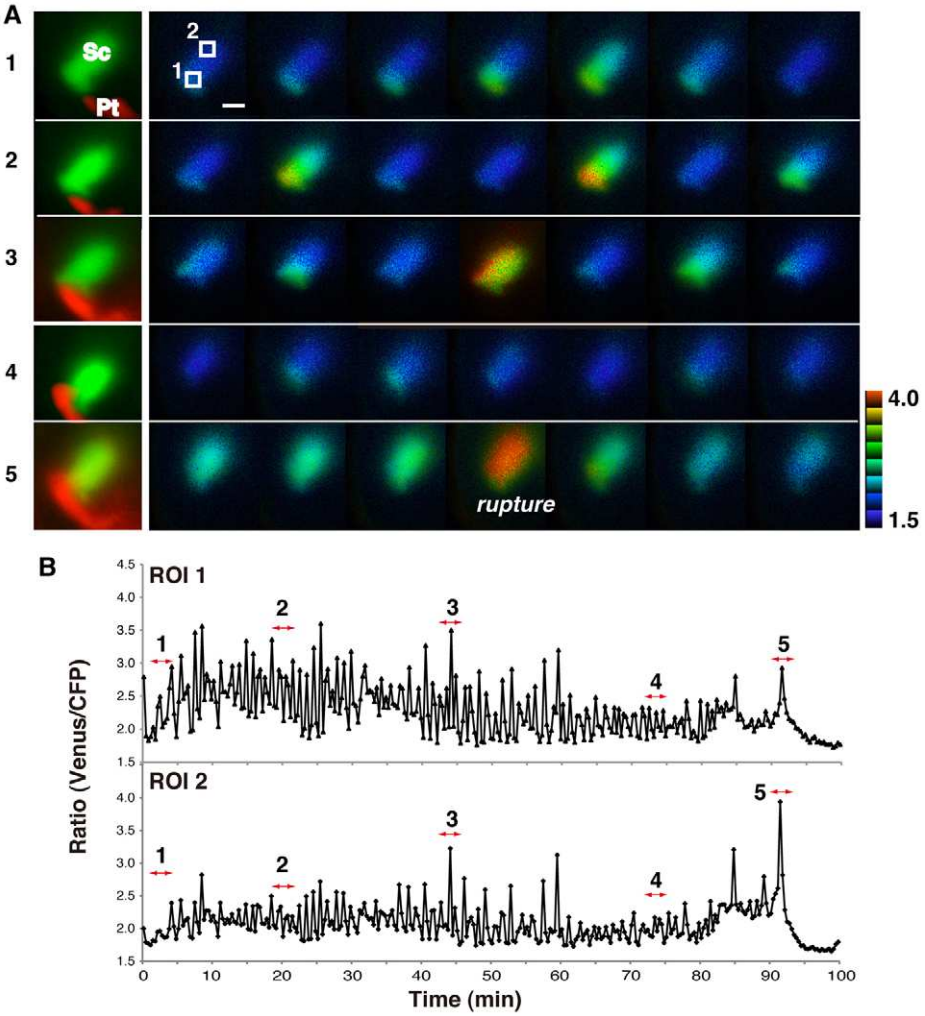

Fig. 4. Ca^{2+} imaging in the synergid cells after pollen tube arrival. pAct1::YC3.60-expressing plant and pAct1::RFP-expressing plant were used. (**A**) Left panels: images of the RFP-labeled pollen tube and Venus-labeled synergid cells. Right panels: ratio images of synergid cells showing pollen tube approach and pollen tube rupture. Ratio images in each panel were taken sequentially at 20-second intervals. Pt, pollen tube; Sc, synergid cells. Scale bar: 10 μm. Squares 1 and 2 in A indicate the micropylar pole and the chalazal pole of the synergid cells, respectively. (**B**) Ratio changes of each region of interest in the synergid cells in A. Numbers in the graph correspond to the image numbers in A. Data were obtained at intervals of 20 seconds.

Sandaklie-Nikolova et al., 2007; Hamamura et al., 2011). In this study, using *Arabidopsis* plants expressing the GFP-based Ca^{2+}-sensing probe YC3.60 in pollen tubes and synergid cells, we visualized Ca^{2+} dynamics in these cells during micropylar guidance and pollen tube reception.

First, we examined whether ovules on the germination medium affected pollen tube growth and $[Ca^{2+}]_{cyt}$ dynamics. We found that pollen tubes repeatedly turned within 150 μm of the micropyles of wild-type ovules, whereas such turning was never observed near *myb98* mutant ovules or in the absence of ovules. In addition, $[Ca^{2+}]_{cyt}$ in pollen tube tips close to wild-type ovules was significantly higher than in those near *myb98* ovules or in the absence of ovules. By contrast, $[Ca^{2+}]_{cyt}$ near the pistil in the presence of wild-type ovule was not different from that in the presence of *myb98* ovules. From the above observations, we can associate the turning of the pollen tubes with an increase in $[Ca^{2+}]_{cyt}$. Thus, we hypothesize that pollen tubes sense some substance produced by wild-type ovules that affects the growth direction and $[Ca^{2+}]_{cyt}$ of the pollen tube. Attractants similar to the *Torenia* LURE-like proteins may also be produced by *Arabidopsis* synergid cells and may affect Ca^{2+} signaling in the pollen tube. Whether this also occurs in *Torenia* is currently unknown. However, $[Ca^{2+}]_{cyt}$ decreased when the pollen tube approached the micropyle or funiculus. The attractants from synergid cells are thought to form a concentration gradient around the micropyle in the germination medium. Our results suggest that the attractants elevate $[Ca^{2+}]_{cyt}$ in the pollen tube at a specific concentration, but at higher concentrations attractants reduce $[Ca^{2+}]_{cyt}$ in the pollen tube. In addition, we observed that there was no significant change in $[Ca^{2+}]_{cyt}$ oscillation and concentration at the tip region before the pollen tubes ruptured, although we detected Ca^{2+} oscillation in the synergid cells. These results indicate that a precise sequence of Ca^{2+} dynamics in the pollen tube may be a prerequisite for pollen tube rupture.

Regarding the interaction between pollen tubes and synergid cells, an increase in pollen tube $[Ca^{2+}]_{cyt}$ was observed at the time of pollen tube rupture. Monitoring revealed an absence of the $[Ca^{2+}]_{cyt}$ increase at the tip region before pollen tube burst and a spreading of the high $[Ca^{2+}]_{cyt}$ area from the tip region to the shank after pollen tube rupture. Recent evidence suggests that the pollen tube receptor-like kinases ANXUR1 and ANXUR2, which are closely related to the receptor-like kinase FERONIA, prevent premature rupture during pollen tube growth (Miyazaki et al., 2009; Boisson-Dernier et al., 2009; Kessler and Grossniklaus, 2011). However, it is currently not known whether Ca^{2+} signaling is involved in transducing a signal after the interaction of ANXUR with a putative ligand from the synergid cells. In addition, in *aca9* mutants, where a predicted Ca^{2+}/calmodulin-activated Ca^{2+} pump is disrupted, pollen tubes normally reach the embryo sac and cease growth, but fail to rupture and release the sperm cells (Schiøtt et al., 2004). Monitoring $[Ca^{2+}]_{cyt}$ dynamics in these *aca9* mutants and other mutants, in which the pollen tube fails to rupture within the ovule, will elucidate the precise role of Ca^{2+} signaling in controlling pollen tube rupture.

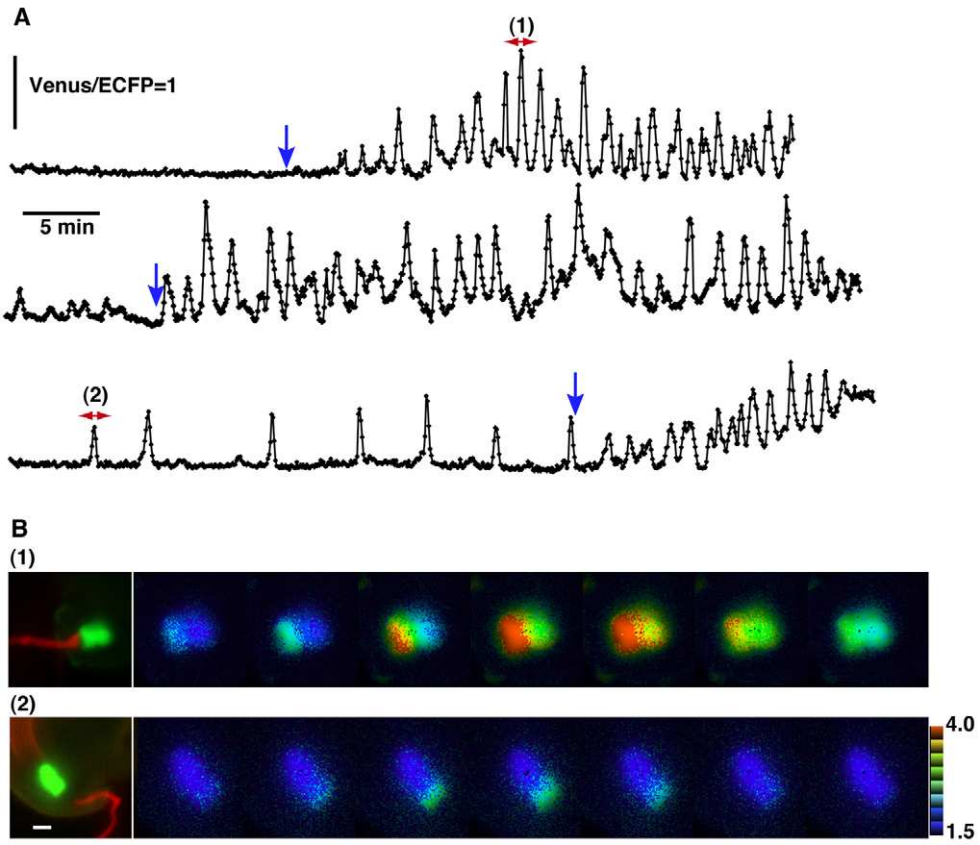

Fig. 5. Relationship between Ca^{2+} oscillation in the synergid cell and the pollen tube approach. pDD2-like::YC3.60-expressing ovules and pAct1::RFP-expressing pollen were used. (**A**) Ratio changes at the micropylar pole of the synergid cells (8 μm × 8 μm). Numbers in the traces correspond to the image numbers in B. The timing of contact of the pollen tube with the synergid cell is the time point when pollen tube temporarily ceases growth (blue arrows). Data were obtained at 5-second intervals. (**B**) Left panels: images of pollen tube (RFP) and synergid cells (Venus) in the ovule. Right panels: ratio images of synergid cells. Ratio images at 5-second intervals are shown. Scale bar: 20 μm.

There was no change in the $[Ca^{2+}]_{cyt}$ of synergid cells until they were approached by the pollen tube after it had entered the ovule. In very few cases, however, $[Ca^{2+}]_{cyt}$ oscillations initiated in the synergid cells before pollen tube reached them. Finally, $[Ca^{2+}]_{cyt}$ in synergid cells reached the maximum level at the time that the pollen tube burst. These $[Ca^{2+}]_{cyt}$ monitoring data suggest that the pollen tube induces $[Ca^{2+}]_{cyt}$ change in the synergid cells, and that Ca^{2+} signaling is involved in pollen tube-synergid cell interactions. In legumes, *Rhizobium* bacteria cause the development of a nodule, which is the site for bacteria and plant to cooperate in the fixation and assimilation of nitrogen (Mylona et al., 1995). *Rhizobium* Nod factors induce $[Ca^{2+}]_{cyt}$ oscillations in root hair cells. It has been reported that the oscillation with a mean period of 60 seconds starts within 10 minutes of Nod factor addition (Ehrhardt et al., 1996). In this study, we observed rare cases where $[Ca^{2+}]_{cyt}$ increase occurred in the synergid cells when the pollen tube was more than 20 μm away. This might suggest the existence of a diffusible signal from pollen tube, which affects $[Ca^{2+}]_{cyt}$ in synergid cells, although contact of the pollen tube with the synergid cells would be necessary for the strong Ca^{2+} oscillation observed later on.

The synergid cell that receives the pollen tube undergoes cell death. It is thought that degeneration of the synergid cell is required for pollen tube entry into the synergid cell and to provide access of the sperm cells to the egg and central cell for fertilization (Punwani and Drews, 2008). Sandaklie-Nikolova et al. proposed a model in which a signaling cascade triggered by the contact of the pollen tube with the synergid cell induces synergid cell death in *Arabidopsis* (Sandaklie-Nikolova et al., 2007). In pathogen-plant interactions, $[Ca^{2+}]_{cyt}$ increase is involved as an early signal in the hypersensitive response (HR) (Levine et al., 1996). The HR involves rapid programmed cell death in the local region surrounding an infection site (Dangl et al., 1996). Therefore, the finding that $[Ca^{2+}]_{cyt}$ increase and oscillation were induced by the interaction of the pollen tube with the synergid cells suggests that synergid cell death occurs downstream of the Ca^{2+} signaling cascade. In this study, when the pollen tube reaches the micropylar pole of the synergid cells, Ca^{2+} oscillations can be observed in all synergid cells after contact with the pollen tube. Furthermore, before pollen tube rupture and cell death of synergid cells occurs, Ca^{2+} oscillations can also be observed in all synergid cells, even in those where subsequently the pollen tube did not rupture. Therefore, it is probable that Ca^{2+} oscillation in the synergid cell is a necessary but not a sufficient condition for pollen tube rupture. During HR, H_2O_2 induces Ca^{2+}-mediated cell death. The pollen tube generates reactive oxygen species (ROS) such as H_2O_2 (Dresselhaus and Márton, 2009), which may also function as signaling molecules in this interaction, inducing $[Ca^{2+}]_{cyt}$ changes upstream of programmed cell death.

In this study, a $[Ca^{2+}]_{cyt}$ increase was observed at the time of pollen tube rupture. Changes in $[Ca^{2+}]_{cyt}$ are thought to be regulated by transporters localized in the membrane of Ca^{2+} storage organelles and the plasma membrane (Sze et al., 2000). Previous electron microscopic studies in pearl millet ovaries reported that Ca^{2+} precipitations with antimonate were observed at the cell wall in the filiform apparatus and the developed ER structure under the cell membrane in synergid cells (Chaubal and Reger, 1992a; Punwani and Drews, 2008). We could not clarify the source of Ca^{2+} for the observed $[Ca^{2+}]_{cyt}$ increase in the synergid cells in this study, so it is still unknown whether the Ca^{2+} is delivered from the extracellular region, the ER, or both. Our $[Ca^{2+}]_{cyt}$ imaging results suggest that signaling molecules from the pollen tube, or physical stimulation by the pollen tube, affect Ca^{2+} dynamics in the synergid cells, and vice versa.

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Competing interests statement
The authors declare no competing financial interests.

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